Effects of Postnatal Exposure to Mixtures of Non-ortho-PCBs, PCDDs, and PCDFs in Prepubertal Female Rats

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There are concerns that postnatal exposure to organochlorines present in breast milk could lead to adverse health effects. We reconstituted four mixtures of aryl-hydrocarbon receptor (AhR) agonists (3 non-ortho polychlorinated biphenyls [PCBs], 6 polychlorinated dibenzodioxins [PCDDs], 7 polychlorinated dibenzofurans [PCDFs], or all 16 chemicals together [referred to as AhRM]) based on their concentrations in breast milk, and examined their effects following exposure by gavage from day 1 until day 20 of age. Female neonates received dosages of AhRM equivalent to 1, 10, 100, or 1000 times the amount consumed by an infant over the first 24 days of life. Other groups received the PCBs, the PCDDs, or the PCDFs at the 1000 level. All rats were sacrificed at 21 days of age. Changes in ethoxyresorufin-o-deethylase hepatic activity, thymus and body weights, and serum thyroxin were linked to the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxic equivalents (TEQ) of the four mixtures (1000× AhRM > PCDDs > PCBs > PCDFs). To test for AhRM antioestrogenic, two additional groups received 1.5 μg/kg of 17α-ethynyl estradiol (EE) with or without the 1000×AhRM. The AhRM had no effect on uterine weight or EE-stimulated uterine growth. The actions of the combined EE and AhRM treatments suggest additive effects in decreasing pentoxyresorufin-0-deethylase activity and spleen weight, but nonadditive/antagonistic effects on adrenal weight and serum thyroxin. In conclusion, (1) 10×AhRM had no detectable effects, (2) TEQ values relate to observed toxicities, even when testing complex mixtures of AhR agonists, and (3) indications of tissue-specific additive and nonadditive/antagonistic effects, but no synergism, were observed when doses of AhRM were increased, or combined with EE.

Key Words: uterotrophic bioassay; prepubertal; rat; mixture; CYP 2B; toxic equivalent; polychlorinated biphenyls; polychlorinated dibenzodioxins; polychlorinated dibenzofurans; breast milk.

Organochlorines are ubiquitous environmental contaminants for which human exposure starts at the gamete stage (Bush et al., 1986; Jarrell et al., 1993; Pauwels et al., 2000; Schecter et al., 1996), followed by in utero (Feeley and Brouwer, 2000; Foster et al., 2000), and the postnatal period. There is concern that exposure to chemicals during the developmentally sensitive in utero and postnatal periods may have long-term adverse health consequences, including increased risk of developing cancers, reproductive problems, learning difficulties, and immune system and thyroid function deficiencies (Aoki, 2001; Golden et al., 1998). During the postnatal period, exposure to organochlorines is more important in breastfed than nonbreastfed infants, since breast milk is an important route for excretion of accumulated lipophylic contaminants (Haines et al., 1998; Lorber and Phillips, 2002). There is limited data available concerning risks associated with exposure to breast milk contaminants (Landrigan et al., 2002; Pronczuk et al., 2002). The beneficial effects of breastfeeding are well recognized, and it has recently been suggested that neurological impairments originating from in utero exposure to organochlorines may be counteracted by breastfeeding (Ribas-Fito et al., 2003). Nevertheless, a better understanding of possible adverse effects resulting from exposure in utero, during the postnatal period, or from the combined periods, is essential to ensure appropriate risk assessment and management, and for regulatory agencies to develop chemical testing strategies adequately predicting outcomes later in life.

Non-ortho polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) are organochlorines present as contaminants in breast milk (Brouwer et al., 1998a; Dewailly et al., 1992; Lorber and Phillips, 2002). These chemicals exert most of their toxic effects through activation of the aryl-hydrocarbon receptor (AhR), and are referred to as AhR agonists (Denison et al., 2002; Safe, 2001). Based on their toxic effects in the liver, thymus gland, and body weight (Safe, 2001), they can be considered among the most toxic organochlorines. The effectiveness of these chemicals to bind the AhR and induce these toxic effects was used to set up the Toxic Equivalent (TEQ) system. This system provides indices of toxicity for AhR agonists relative to TCDD, and simplifies risk assessment of mixtures (Safe, 1994; van Leeuwen et al., 2000). In addition,

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AhR agonists have the potential to promote cancer and affect the immune, nervous, reproductive, and endocrine systems (Aoki, 2001; Baccarelli et al., 2002; Petroff et al., 2001b; Tian et al., 2002). Among endocrine effects, modulation of the estrogen receptor (ER) and thyroid hormone systems are being extensively studied. AhR agonists have been shown to act as antiestrogens, not by competing for the ligand domain of the ER, but by downregulating ER, increasing the rate of estradiol-17β metabolism, inhibiting various growth factors and cell cycle enzymes (Chen et al., 2001), mainly through crosstalk between AhR and ER pathways (Reen et al., 2002; Safe, 1999). Liganded AhR complexes interact with DNA dioxin response elements near promoter regions activated by ER and thus induce antiestrogenic effects (reviewed by Safe, 1999). They could also interact with unliganded ER to induce estrogenic effects (Ohtake et al., 2003). AhR agonists affect multiple components of the thyroid system, including displacement of thyroid hormones from the carrier protein transthyretin to decrease deiodinase and increase glucuronidase enzyme activities (Brouwer et al., 1998b; Khan et al., 2002; Vansell and Klaassen, 2002a,b; Wade et al., 2002).

Very few studies have tested the effects of reconstituted breast milk contaminant mixtures in neonates, and at exposure levels similar to humans. Parkinson et al. (1980) observed that a reconstituted breast milk mixture of 13 ortho-PCBs was seven times more potent for stimulating hepatic aryl hydroxylase activity, compared to the more complex commercial PCB mixture Kanecolor 500. We found that postnatal exposure to high doses of the most abundant breast milk contaminants (PCBs, p,p’-dichlorodiphenylchloroethane [DDT], p,p’-dichlorodiphenyl dichloroethene [DDE], or to TCDD), promoted the development of methylnitrosourea-induced mammary lesions in the rat (Desaulniers et al., 2001). To continue testing reconstituted mixtures of breast milk contaminants, our objective for the current study is to describe dose-response effects in 21-day-old female Sprague-Dawley rats following postnatal exposure to mixtures of non-ortho PCBs, PCDDs, and PCDFs. The mixtures were prepared according to the amount of these contaminants present in breast milk (Brouwer et al., 1998a; Dewailly et al., 1992; Lober and Phillips, 2002). As classical indicators for effects of AhR agonists, we assessed changes in organ weights, three reference points to the TEQ system (hepatic cytochrome P450 [CYP] 1A1 induction, thymus atrophy, and reduction in body weight [Safe, 2001]) and effects on CYP2B and 3A as other indicators of hepatic exposure. In contrast to CYP1A1, which is regulated through the AhR, the expression of CYP2B and 3A are regulated through heterodimerization of the retinoid X receptor with either the nuclear orphan constitutively active receptor (CAR), or the pregnane X receptor, respectively (Cai et al., 2002; Masahiko and Honkakoski, 2000; Sueyoshi and Negishi, 2001; Ueda et al., 2002; Zelko and Negishi, 2000). Endocrine effects were assessed by measuring serum corticosterone, pituitary luteinizing hormone (LH), serum thyroxin and the pituitary thyroid stimulating hormone (TSH) as indicators of thyroid function (Brouwer et al., 1998b; Khan et al., 2002; Vansell and Klaassen, 2002a,b; Wade et al., 2002). In addition, the antiestrogenic potential of the AhR agonist mixture (AhRM) was investigated by testing its effect on uterine growth and its ability to counteract the uterotrophic effect of 17α-ethynyl-estradiol (EE). The ability of uterine cells to proliferate in response to estrogens is the basis for the rodent uterotropic bioassay. This test, although not very sensitive, is considered the “gold standard” test for estrogenicity, because it takes into account the effects of metabolism, serum binding, and pharmacokinetics (Gray et al., 1997; Odum et al., 1997). It is intended to be used by regulatory agencies to identify the in vivo activities of suspected estrogen agonists or antagonists (Kanno et al., 2001; Padilla-Banks et al., 2001).

MATERIALS AND METHODS

Animal treatment. Four mixtures were prepared and included 3 non-ortho-chlorinated PCBs, 6 PCDDs, 7 PCDFs, or all 16 of these chemicals together, which we will refer to as AhRM, respectively (Table 1). The amounts of each chemical were calculated to achieve, over the exposure period, a cumulative dose representing the amount that a human baby would have consumed over its first 24 days of life. The calculations were based on milk fat concentrations of each chemical present in breast milk samples collected in 1989–1990 from Caucasian women in southern Quebec (Dewailly et al., 1992), a newborn consumption rate of 120 ml breast milk/kg body weight per day (Ayotte et al., 1995), and a milk fat content of 3.7% (Frank et al., 1988).

At one day of age, female Sprague-Dawley rats (Charles River, St-Constant, QC) were randomly assigned to 18 fostering dams with five to eight female neonates per dam. To randomize the litter effect, the neonates within a litter were assigned to different treatment groups (10 to 13 neonates per group). The experiment included nine separate treatment groups: water, corn oil (vehicle), AhRM at dosages equivalent to 1, 10, 100, or 1000 times the amount that an infant would have consumed over its first 24 days of life, and the three chemical family components of the 1000×AhRM which include the non-ortho PCBs, PCDDs, and PCDFs. The neonates were identified and exposed to the vehicle or the mixtures by gavage at ages 1, 5, 10, 15, and 20 days, each dose (5 ml of mixture per kg bw) representing 5 days of ingestion (Fig. 1). Two additional groups were used to test for antiestrogenic effects of the 1000X-AhRM. The first group (n = 10) received corn oil (the vehicle for the AhRM) at 1, 5, 10, 15, and 20 days of age and a daily dose of 17α-ethynyl estradiol (EE; CAS No. 57–63–6; 0.5 μg/kg, corn oil as vehicle) injected sc in the morning on days 18, 19 and 20 of age. The second group included eleven rats receiving the same doses of EE plus AhRM as described previously (Fig. 1). All rats were sacriﬁced at 21 days of age.

Radioimmunoassays. Serum thyroxin and corticosterone levels were determined using commercial 125I radioimmunoassay (RIA) kits (ICN Biomedical, Costa Mesa, CA), while pituitary LH and TSH contents were determined by RIA as previously described (Desaulniers et al., 1999). Briefly, the pituitary glands were sonicated in 200 μl of 0.05 M phosphate-buffered saline, 1% BSA and protease inhibitors (1 mM EDTA, 1.0 μM pepstatin A, 10 μM E-64, 17 μM 2-deoxy-2-deoxy-a-lyeulinuracil acid and 1.5 μg/ml aprotinin; Sigma Chemicals, St. Louis, MO) and the homogenates were centrifuged (1300 × g, 30 min, 4°C), to recuperate the supernatant. The sonication/centrifugation procedure was repeated twice, resulting in a final volume of 0.6 ml from which hormones were measured. Pituitary hormone preparations for iodination, standard curves, and primary antisera were rLH-I-9 (AFP-10250C), rLH-RP-3 (AFP-7187B), and rLH-S-11 for LH and rTSH-I-9 (AFP-11542B), rTSH-RP-3 (AFP-5512B), and rTSH-RIA-6 for TSH, respectively (all provided by the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases). The sensitivity of the LH, TSH, thyroxin, and corticosterone assays were 0.02 ng/tube, 0.04 ng/tube, 0.63 μg/dl.
and 25 ng/ml, respectively, and the interassay coefficients of variation were 6.9, 6.2, 7.4, and 13%, respectively.

**Hepatic cytochrome P450 enzyme assays.** Hepatic CYP1A1 and CYP2B1/2 activities in 10,000×g supernatants were examined by determining the activities of ethoxyresorufin-o-deethylation (EROD), pentoxyresorufin-o-deethylation (PROD), and benzyloxyresorufin-o-deethylation (BROD), according to the method of Burke et al. (1985). While PROD and BROD activities have classically been considered indicators of CYP2B1/2 and 3A activities (Burke et al., 1985; Chen and Eaton, 1991; Namkung et al., 1998), respectively, they have been shown to predominantly reflect CYP2B activities (Nerurkar et al., 1993). CYP enzyme activities were calculated from the slope of the linear portion of the reaction.

**Analysis of hepatic cytochrome P450 protein content by Western blot.** Liver homogenates, prepared in 2.5 volumes of cold Tris-KCl (0.5M/1.15%; pH 7.4) were centrifuged (10,000×g, 20 min) to obtain a post mitochondrial fraction (PMF) that was further centrifuged (105,000×g, 1 h). The pellets were resuspended in 0.5M Tris/HCl (pH 6.8) and centrifuged again (105,000×g, 1 h) to obtain the microsomal preparations. The protein concentration of the microsomal solution was measured by the method of Bradford (1976). Microsomal preparations were diluted 1:1 with loading buffer (0.5M Tris/HCl pH 6.8, 10% glycerol, 0.5% sodium dodecyl sulfate [SDS]), and the proteins were resolved (50 min at 150V) on 4–20% gradient precast gels (ICN Biomedicals, Aurora, OH). The proteins were electro-transferred (100 V at 0EC) onto nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol, pH 8.3). The membranes were treated with SuperBlock (Pierce Chemical Co., Rockford, IL) and 0.1% Tween20 in phosphate-buffered saline (PBS) to block nonspecific binding sites and incubated with the primary

![FIG. 1.](https://academic.oup.com/toxsci/article-abstract/75/2/468/1655912) Experimental design. Female neonates were exposed to the mixtures by gavage (5 ml/kg bw) at day 1 of age (a cumulative dose for days 0, 1, 2, 3, and 4), day 5 (for days 5, 6, 7, 8, and 9), day 10 (for days 10, 11, 12, 13, 14), day 15 (for days 15, 16, 17, 18, 19), and 20 days (for days 20, 21, 22, 23, and 24). In addition, between 0800–0900 h on day 18, 19, and 20 of age, two groups (oil, 1000X) were weighed and injected sc with 17α-ethynyl estradiol (EE; 0.5 μg/kg). All rats were sacrificed at 21 days of age.

**TABLE 1**

**Concentrations of PCB, PCDF, and PCDD Isomers in the 1000× AhRM**

<table>
<thead>
<tr>
<th>Family of xenobiotics</th>
<th>Selected isomers</th>
<th>Milk fat (ng/ml)a</th>
<th>Target (μg/ml)b</th>
<th>Analyzed (μg/ml)c</th>
<th>TEFd</th>
<th>TEQ (ng/g)f</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-ortho PCBs</td>
<td>3,3’,4,4’-TetraCB</td>
<td>0.008</td>
<td>0.036</td>
<td>0.026</td>
<td>0.0001</td>
<td>0.0026</td>
</tr>
<tr>
<td></td>
<td>3,3’,4,4’,5-PentaCB</td>
<td>0.08</td>
<td>0.355</td>
<td>0.332</td>
<td>0.1</td>
<td>33.2</td>
</tr>
<tr>
<td></td>
<td>3,3’,4,4’,5,5’-HexaCB</td>
<td>0.033</td>
<td>0.147</td>
<td>0.114</td>
<td>0.01</td>
<td>1.14</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>0.121</td>
<td>0.538</td>
<td>0.472</td>
<td>—</td>
<td>34.3426</td>
</tr>
<tr>
<td>PCDFs</td>
<td>2,3,7,8-TetraCDF</td>
<td>0.006</td>
<td>0.027</td>
<td>0.0157</td>
<td>0.1</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td>2,3,4,7,8-PentaCDF</td>
<td>0.005</td>
<td>0.022</td>
<td>0.0246</td>
<td>0.5</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>1,2,3,4,7,8-HexaCDF</td>
<td>0.003</td>
<td>0.013</td>
<td>0.0177</td>
<td>0.1</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>1,2,3,6,7,8-HexaCDF</td>
<td>0.002</td>
<td>0.009</td>
<td>0.0116</td>
<td>0.1</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>2,3,4,6,7,8-HexaCDF</td>
<td>0.001</td>
<td>0.004</td>
<td>0.005</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1,2,3,4,6,7,8-HeptaCDF</td>
<td>0.005</td>
<td>0.022</td>
<td>0.0214</td>
<td>0.01</td>
<td>0.214</td>
</tr>
<tr>
<td>OctaCDF</td>
<td></td>
<td>0.001</td>
<td>0.004</td>
<td>0.003</td>
<td>0.0001</td>
<td>0.0003</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>0.023</td>
<td>0.101</td>
<td>0.099</td>
<td>—</td>
<td>17.5143</td>
</tr>
<tr>
<td>PCDDs</td>
<td>2,3,7,8-TetraCDD</td>
<td>0.002</td>
<td>0.009</td>
<td>0.0024</td>
<td>1</td>
<td>2.4</td>
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<tr>
<td></td>
<td>1,2,3,7,8-PentaCDD</td>
<td>0.005</td>
<td>0.022</td>
<td>0.0165</td>
<td>1</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>1,2,3,4,7,8-HexaCDD</td>
<td>0.035</td>
<td>0.155</td>
<td>0.167</td>
<td>0.1</td>
<td>16.7</td>
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<td></td>
<td>1,2,3,7,8,9-HexaCDD</td>
<td>0.006</td>
<td>0.027</td>
<td>0.0235</td>
<td>0.1</td>
<td>2.35</td>
</tr>
<tr>
<td></td>
<td>1,2,3,4,6,7,8-HeptaCDD</td>
<td>0.041</td>
<td>0.182</td>
<td>0.179</td>
<td>0.01</td>
<td>1.79</td>
</tr>
<tr>
<td>OCDD</td>
<td></td>
<td>0.132</td>
<td>0.586</td>
<td>0.453</td>
<td>0.0001</td>
<td>0.0453</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>0.221</td>
<td>0.981</td>
<td>0.8414</td>
<td>—</td>
<td>39.7853</td>
</tr>
<tr>
<td>Grand total</td>
<td></td>
<td>0.365</td>
<td>1.62</td>
<td>1.412</td>
<td>—</td>
<td>91.6422</td>
</tr>
</tbody>
</table>

aThe concentrations of each chemical included in the mixture are based on the levels found in breast milk fat (Dewailly et al., 1992).

bTarget concentrations were those that we wanted to achieve in the mixtures. They were calculated so that rats exposed by gavage with 5.0 ml/kg of mixture would receive 1000 times the amount ingested by a human baby. Calculations are based on milk fat concentrations for each organochorines, a newborn consumption rate of 120 ml breast milk/kg body weight per day (Ayotte et al., 1995), with a milk fat content of 3.7% (Frank et al., 1988). Dosing mixtures at the 1×, 10×, and 100× level were prepared by diluting the 1000× mixture.

cAnalyzed concentrations indicate the congener concentrations in the 1000× dosing solution, measured in a blind test (Wellington Laboratories, Guelph, ON, Canada).

dThe Toxic Equivalency Factor is an index of toxicity relative to TCDD for which TEF = 1 (Van den Berg et al. 1998).

eToxic Equivalents (TEQ) were derived from multiplying the analyzed concentration of each chemical by the TEF values. The sum of the TEQ values attributed to non-ortho PCBs, PCDFs, PCDDs, and the complete mixture are presented. The total dose administered to the 1000X group, was 51× ng TCDD-TEQ, or 1.1 g/kg bw. The conversion to μg/kg is based on the average weight of the rats from the 1000× group at 20 days of age (45 g). The total dose was derived by multiplying the TCDD-TEQ of the mixture by the total volume of solution administered to each rat from day 1 until day 20.

fThe Toxic Equivalency Factor is an index of toxicity relative to TCDD for which TEF = 1 (Van den Berg et al. 1998).
antibody (1 h, RT; all from Gentest Corporation, Woburn, MA). Blots were then washed with 0.1% Tween20 in PBS, incubated with an alkaline phosphatase conjugated secondary antibody and visualized using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich Ltd., Oakville, ON, Canada). Signal intensity of the samples and standards (CYP1A1, In Vitro Technologies, Inc. Baltimore, MD; CYP3A2, and CYP2B1, Gentest Corporation, Woburn, MA) were quantified using an imaging densitometry system (BioRad Model GS-670; Molecular Analyst/PC software, BioRad Laboratories Inc., Mississauga, ON, Canada). The results were corrected for the amount of protein loaded on the gel and are expressed as the ratio of sample density/standard density.

**Determination of hepatic CYP2B1, CYP3A2, and ERα mRNA abundance.** The hepatic mRNA abundance of CYP2B1, CYP3A2, and ERα was determined by quantitative real-time reverse transcription (RT)-PCR method. Total RNA was isolated from rat liver tissue samples using the RNeasy mini kit and the RNase-free DNase set (Qiagen, Mississauga, ON, Canada). RT of the total RNA was performed at 37°C for 1 h in 20 µl of the reaction mixture containing 2 µg of total RNA, 0.5 mM dNTPs, 10 units of recombinant RNase inhibitor, 1 µM oligo (dT) primer and 4 units reverse transcriptase (Qiagen Omniscript reverse transcriptase kit) and inactivated by heating (95°C, 5 min). To test for residual DNA contamination, negative control samples did not include the reverse transcriptase. The resulting cDNA was amplified by quantitative real-time PCR analysis.

The quantitative real-time PCR (c)ycler iQ system, BioRad, Mississauga, ON, Canada) was performed in 25 µl of reaction mixture containing QuantiTect SYBR Green PCR Master Mix (1X, BioRad, Mississauga, ON, Canada), 4 µl of RT products (80 ng total RNA), and 0.3 µM primers (forward and reverse). The temperature program included 15 min at 95°C; then 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. The PCR primers (forward and reverse), designed in accordance with published rat gene sequences (Fujii-Kuriyama et al., 1982; Miyata et al., 1994; Nudel et al., 1983; Spreafico et al., 1992), were CAACTAGCCTGTTGCC and GTGTGTTGCC for β-actin; TTCAGCTCTCACAGTGA and AAGAAGCACAGGTGTG for CYP3A2; TATGTCCTGGAATGGGGAAC and CCGTTCTCTCCACTCTCTTCT for CYP2B1; and GCCGCCTACGAGGTCA and GACCTTAAGTGATCGTCCAGCT for ERα. The amplification of a single specific DNA band of the correct size was verified by gel electrophoresis, and the presence of a single melting curve. The amount of PCR product was quantified by comparing the threshold cycle of each sample to the standard curve established with threshold cycles of serially diluted quantities of purified cDNA of the same genes. The results were then normalized relative to the abundance of β-actin mRNA present in each sample.

**Statistical analysis.** The effects of treatments on body weight were analyzed using the software SAS (release 8.02; SAS Institute Inc., Cary, NC, 2001) with the following repeated measure model:

\[ y_{ij} = \mu + T_i + A_{ij} + P_k + (TP)_{ik} + \epsilon_{ijk} \]

where \( T_i \) represents the \( i^{th} \) treatment group, \( A_{ij} \) is the \( i^{th} \) animal nested in the \( i^{th} \) treatment group, \( P_k \) is the \( k^{th} \) time period, and \( \epsilon_{ijk} \) is the weight of the \( j^{th} \) animal in the \( i^{th} \) treatment group at time \( k \). The error term used for testing treatment differences, and \( \epsilon_{ijk} \) represents error associated with period differences as well as the interaction between period and treatment differences. All other statistical analyses were performed with the software JMP (SAS Institute Inc., 1998) by applying one-way ANOVA followed by the Tukey-Kramer test to identify significantly different means. The analysis of data which tested the effects of EE and 1000X-AhRM were performed using two way ANOVA considering the effects of the mixture, EE, and their interaction. Homogeneity of variances and normality of the data were initially verified by O’Brien, Brown-Forsyth and Shapiro-Wilk Goodness-of-fit tests (SAS Institute Inc., 1998). The nonparametric Wilcoxon/Kruskal Wallis tests were used when normality of data and/or equal variances were not reached. In all cases, \( p \leq 0.05 \) was considered to be a significant effect, whereas \( p > 0.1 \) was considered as a tendency of effect.

**RESULTS**

The effects of treatment on body weight were not statistically significant, but they were inversely related to the administered TEQ doses for the different groups (Tables 2 and 3). The liver/body weight ratio was significantly increased with 1000×-AhRM, PCB, and PCDD treatments (Tables 2 and 3), while only the 1000×-AhRM treatment decreased thymus and adrenal weights (Table 2). The 1000×-AhRM had no effects on brain weights, but it increased the brain/body weight ratios

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
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<tbody>
<tr>
<td><strong>TCDD-TEQ Doses for the AhRM and Dose-Response Effects on Organ Weights and Serum Thyroxin</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TCDD-TEQ (ng/rat)</th>
<th>Water</th>
<th>Oil</th>
<th>1×</th>
<th>10×</th>
<th>100×</th>
<th>1000×</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>51.6 ± 1.7</td>
<td>52.7 ± 1.0</td>
<td>52.1 ± 1.6</td>
<td>50.2 ± 1.2</td>
<td>51.8 ± 1.1</td>
<td>47.2 ± 1.3</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>2.22 ± 0.06</td>
<td>2.27 ± 0.05</td>
<td>2.26 ± 0.09</td>
<td>2.13 ± 0.06</td>
<td>2.29 ± 0.05</td>
<td>2.35 ± 0.06</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>4.30 ± 0.22</td>
<td>4.30 ± 0.03</td>
<td>4.33 ± 0.06</td>
<td>4.25 ± 0.06</td>
<td>4.42 ± 0.05</td>
<td>4.98 ± 0.06</td>
</tr>
<tr>
<td>Both kidneys (mg)</td>
<td>263 ± 9</td>
<td>292 ± 9</td>
<td>289 ± 19</td>
<td>259 ± 10</td>
<td>279 ± 15</td>
<td>246 ± 12</td>
</tr>
<tr>
<td>Both adrenals (mg)</td>
<td>16.16 ± 0.77</td>
<td>17.78 ± 0.71</td>
<td>16.83 ± 0.49</td>
<td>15.11 ± 0.48</td>
<td>15.65 ± 0.62</td>
<td>14.63 ± 0.56</td>
</tr>
<tr>
<td>Thymus (mg)</td>
<td>0.032 ± 0.002</td>
<td>0.034 ± 0.001</td>
<td>0.033 ± 0.001</td>
<td>0.030 ± 0.001</td>
<td>0.030 ± 0.001</td>
<td>0.031 ± 0.001</td>
</tr>
<tr>
<td>Brain (g)</td>
<td>1.45 ± 0.01</td>
<td>1.44 ± 0.02</td>
<td>1.44 ± 0.02</td>
<td>1.43 ± 0.02</td>
<td>1.43 ± 0.01</td>
<td>1.40 ± 0.02</td>
</tr>
<tr>
<td>Thyroxin (µg/dl)</td>
<td>3.66 ± 0.27</td>
<td>3.74 ± 0.16</td>
<td>3.29 ± 0.21</td>
<td>3.75 ± 0.28</td>
<td>3.31 ± 0.25</td>
<td>2.37 ± 0.13</td>
</tr>
</tbody>
</table>

**Note.** \( n = 10 \) to 13 per group; mean ± SE; ns = no significant differences or tendencies; \( o/b \) = organ weight (g)/body weight (g) ratio multiplied by 100. \( a,b \)Means with different letters are significantly different (\( p < 0.05 \)), with the largest numbers identified by the superscript "a". There were no effects on the mammary gland area, the weight of the uterus, ovaries, and pitable glands.
Serum thyroxin concentrations were significantly lower in the 1000×-AhRM group (Tables 2 and 3), although none of the mixture treatments affected serum corticosterone, LH and TSH contents.

A significant increase in EROD activity was detected in the 100× group (p < 0.05), while the largest response was observed in the 1000×-AhRM group (Fig. 2). The PCB, PCDD, and PCDF treatments increased EROD activity with the smallest induction observed in the PCDF group. Although the PCDD group received a TCDD-TEQ dose 55% lower than that of the 1000×-AhRM group, there were no significant differences in EROD activities between the two groups (Fig. 2A). PROD activity in the 1000×-AhRM group was reduced below the control level (p = 0.007, Fig. 2B). BROD activity was significantly increased in the 100X AhRM, PCDF, and PCDD groups (p < 0.05, Fig. 2C). In light of the large coefficients of variation for PROD and BROD data at high dosages, we verified the effects of 1000×-AhRM by measuring the hepatic CYP1A1, CYP2B1, and CYP3A2 protein and mRNA levels (Table 4). Protein and mRNA analyses confirmed the suppression of CYP2B1 by 1000×-AhRM. This effect on CYP2B1 mRNA was also detectable at the 100× dose (p < 0.05). The 1000×-AhRM significantly increased messenger RNA abundance and protein content of CYP1A1, but not of CYP3A2. There were no significant correlations between enzyme activities and associated protein levels. The hepatic ERα mRNA abundance was reduced by 1000×-AhRM.

Figure 3 shows a regression analysis between the TCDD-TEQ doses of the AhRM relative to hepatic EROD activities. TCDD-TEQ values were derived by multiplying the TCDD-TEQ of each mixture by the cumulative volume of solution administered to each rat from day 1 until day 20. The body weight at day 20 was used to transform the TCDD-TEQ dose on a per kg basis. The expected EROD activity for the PCDD group, calculated using the regression and the average TCDD-TEQ dose for that group, is significantly lower (p < 0.0001) than the experimental EROD activity (Fig. 2). Perhaps this is an indication of increased nonadditive/antagonistic effects among chemicals of the more complex AhRM. A lowest observable effect level (LOEL) of 15 ng/kg AhRM was calculated based on EROD activity at the 95% upper bound level (0.0799 nmol/min/mg) of the no observable effect level (NOEL, based on the 1×-AhRM group). Note that the 95% upper bound level for the combined water, corn oil, and 1×-AhRM groups is similar (0.0793 nmol/min/mg).

The antiestrogenicity of 1000×-AhRM was studied by testing its ability to attenuate the EE-induced uterotrophic effect (Table 5). This test also studied the combined effects of EE and 1000×-AhRM treatments on other endpoints. As expected the EE treatment increased uterine weight, but the 1000×-AhRM did not reduce this effect. The EE treatment slightly increased pituitary and brain weights. The results from Table 5 confirmed those of Tables 2 and 3, which indicated that AhRM increased liver weight, but decreased the weight of the spleen and thyroxin. Interestingly, there are indications of additive (spleen) and nonadditive/antagonistic effects (kidneys, adrenals, serum thyroxin; Table 5, Fig. 4C). The 1000×-AhRM reduced the weight of the spleen, which was further reduced by the EE treatment. While 1000×-AhRM attenuated the EE-induced reduction in kidney weight, it decreased adrenal weight but this effect was prevented by EE. The decrease in adrenal weight induced by 1000X-AhRM was not associated with significant changes in serum corticosterone level (control: 72 ± 11 ng/ml; EE: 50 ± 8 ng/ml; 1000X-AhRM, 78 ± 13 ng/ml; 1000X-AhRM + EE, 61 ± 13 ng/ml). Serum thyroxin was reduced by 1000×-AhRM, but this effect was counteracted by EE (Fig. 4C). In addition, EE slightly lowered pituitary TSH, but significantly decreased pituitary LH, hepatic PROD and BROD activities (Fig. 4).
DISCUSSION

The dosages of organochlorines administered in this study were based on the concentrations found in Canadian breast milk (Dewailly et al., 1992), and fortunately produced relatively small responses at the dose 100×. These results are consistent with our findings from an experiment conducted concurrently showing no long term effects of 1000×-AhRM on time to vaginal opening, length of estrous cycle, mammary gland development, and methylxynitosourea-induced mammary tumor development (Desaulniers et al., manuscript in preparation).

In the current study, effects on EROD activity, body weight, serum thyroxin, and liver weight followed additive patterns with the most important changes induced by 1000×-AhRM, and to a lesser extent by the PCDD, PCB, and PCDF mixtures. These results confirm that the TEQ system permits the derivation of TEQ values that relate to the observed toxicity, even when testing complex mixtures of AhR agonists. The PCDD mixture, however, induced more EROD activity than what could have been predicted from the AhRM (Fig. 3). This suggests that there may be nonadditive/antagonistic interactions among AhR agonists, and an overestimation of risks based on the TEQ derived from complex mixtures. Similarly, Parkinson et al. (1980) found that a reconstituted breast milk mixture of 13 ortho-PCBs was seven times more potent in stimulating hepatic aryl hydroxylase activity than the more complex commercial PCB mixture Kanechlor 500. No synergistic effects were observed in our study. The number of cellular pathways altered by exposure to chemicals, and pharmacokinetic interactions, are factors determining if effects are additive, antagonistic, or synergistic (Chu et al., 2001; Lee et al., 2002; Petruslis and Bunce, 2000; van Birgelen et al., 1994a,b). Supra-additive (Burgin et al., 2002) or synergistic effects are infrequent findings, which usually result from exposure to mixtures of unrelated chemicals with different modes of action that may regulate the same endpoint (Abbott et al., 1994; Jensen et al., 2000; van Birgelen et al., 1996a,b). In this

![FIG. 2. Hepatic ethoxy (EROD) (A), pentoxy (PROD) (B), and benzylxoy resorufin-o-deethylase (BROD) (C) activities (n = 10 to 13) following postnatal oral exposure to water, oil (vehicle), AhRM in increasing doses (1×, 10×, 100×, and 1000×), and its chemical family components (non-ortho PCBs, PCDDs, and PCDFs) at the 1000× level. Means with different letters are significantly different (p < 0.05). *Significantly smaller, or ** greater, than oil (t-test, p < 0.05). Coefficients of variation are provided within parenthesis.]

### TABLE 4
Effects of the AhR Agonist Mixture on Hepatic CYP1A1, CYP2B1, and CYP3A2, Protein and mRNA Levels, and ERα mRNA Levels (Mean ± SE)

<table>
<thead>
<tr>
<th>Protein (density ratio sample/standard)</th>
<th>Oil (control)</th>
<th>10×</th>
<th>100×</th>
<th>1000×</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>0.45 ± 0.05</td>
<td>NA</td>
<td>NA</td>
<td>1.20 ± 0.09*</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>0.31 ± 0.01</td>
<td>NA</td>
<td>NA</td>
<td>0.22 ± 0.01*</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>0.17 ± 0.02</td>
<td>NA</td>
<td>NA</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>mRNA (attomolar/μg RNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyp1A1</td>
<td>11.3 ± 2.0</td>
<td>18.8 ± 2.3</td>
<td>74.2 ± 15*</td>
<td>192 ± 40*</td>
</tr>
<tr>
<td>cyp2B1</td>
<td>1115 ± 100</td>
<td>1191 ± 196</td>
<td>729 ± 171*</td>
<td>632 ± 153*</td>
</tr>
<tr>
<td>cyp3A2</td>
<td>752 ± 109</td>
<td>NA</td>
<td>NA</td>
<td>743 ± 201</td>
</tr>
<tr>
<td>ERα</td>
<td>1.92 ± 0.91</td>
<td>2.07 ± 1.29</td>
<td>0.64 ± 0.30</td>
<td>0.25 ± 0.05*</td>
</tr>
</tbody>
</table>

Note. NA, not analyzed.
*Significantly different from control, p ≤ 0.05.
context, the TCDD-TEQ system, based mostly on interactions with a single receptor (i.e., AhR), has recognized limitations (Safe, 2001) and cannot correlate with all toxic effects such as estrogenicity, neurotoxicity, oxidative stress, and reproductive and developmental effects (Burgin et al., 2002; Li and Hansen, 1996). The importance of this system should, however, not be minimized as it clearly provides reliable indicators of toxicity, as shown in the present investigations.

In contrast to EROD induction, PROD and BROD activities showed no clear additive effects among the PCB, the PCDD,

![Regression analysis between EROD hepatic activities and TCDD-TEqs in AhRM treated rats](image)

**TABLE 5**

Effects of 1000×-AhRM and 17α-Ethynyl-Estradiol (EE) on Organ Weights

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Uterus (wt weight, mg)</th>
<th>Pituitary gland (mg)</th>
<th>Brain (g)</th>
<th>Liver (g)</th>
<th>Spleen (mg)</th>
<th>Both kidneys (mg)</th>
<th>Both adrenals (mg)</th>
<th>Thymus (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oil</td>
<td>Oil + EE</td>
<td>1000×</td>
<td>1000× + EE</td>
<td>EE</td>
<td>1000×</td>
<td>1000× × EE</td>
<td>1000× × EE</td>
<td>1000× × EE</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>52.7 ± 1.0a</td>
<td>50.7 ± 1.5b</td>
<td>47.2 ± 1.3b</td>
<td>49.2 ± 1.4c</td>
<td>ns</td>
<td>0.009</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Uterus (wt weight, mg)</td>
<td>32.3 ± 1.4a</td>
<td>75.4 ± 5.9a</td>
<td>30.5 ± 0.9b</td>
<td>69.6 ± 1.5c</td>
<td>&lt; 0.0001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Pituitary gland (mg)</td>
<td>0.061 ± 0.003b</td>
<td>0.148 ± 0.009a</td>
<td>0.065 ± 0.003b</td>
<td>0.143 ± 0.006c</td>
<td>&lt; 0.0001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Brain (g)</td>
<td>1.44 ± 0.02a</td>
<td>1.48 ± 0.01a</td>
<td>1.40 ± 0.02a</td>
<td>1.46 ± 0.01c</td>
<td>0.005</td>
<td>0.08</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>2.75 ± 0.04a</td>
<td>2.93 ± 0.07a</td>
<td>2.99 ± 0.07a</td>
<td>2.99 ± 0.08a</td>
<td>ns</td>
<td>0.02</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>4.30 ± 0.03a</td>
<td>4.25 ± 0.08a</td>
<td>4.98 ± 0.06a</td>
<td>4.95 ± 0.09a</td>
<td>&lt; 0.0001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Both kidneys (mg)</td>
<td>292 ± 9a</td>
<td>263 ± 12a</td>
<td>246 ± 12a</td>
<td>231 ± 7a</td>
<td>0.04</td>
<td>0.0005</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Both adrenals (mg)</td>
<td>565 ± 23a</td>
<td>578 ± 20a</td>
<td>607 ± 14a</td>
<td>606 ± 17a</td>
<td>0.04</td>
<td>ns</td>
<td>0.05</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Thymus (mg)</td>
<td>17.78 ± 0.71a</td>
<td>16.95 ± 0.79a</td>
<td>14.65 ± 0.56a</td>
<td>17.51 ± 0.55a</td>
<td>0.1</td>
<td>0.06</td>
<td>0.01</td>
<td>0.007</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>0.034 ± 0.001a</td>
<td>0.033 ± 0.001a</td>
<td>0.031 ± 0.001a</td>
<td>0.036 ± 0.001b</td>
<td>0.03</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>0.51 ± 0.02a</td>
<td>0.49 ± 0.02a</td>
<td>0.46 ± 0.02a</td>
<td>0.43 ± 0.02a</td>
<td>0.007</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Note. n = 10 to 13 per group; mean ± SE; ns = no significant differences or tendencies; o/b = organ weight (g)/body weight (g) ratio multiplied by 100.

Means with different letters are significantly different (p < 0.05), with the largest numbers identified by the superscript “a”. There were no effects on the mammary gland area and ovaries.
and the PCDF groups. Moreover, the 1000×-AhRM decreased PROD activities, CYP2B protein content, and mRNA abundance below the control levels. Others observed antagonistic effects, but of lower magnitude than in the current study, on PROD and/or BROD activities following coadministration of TCDD (or other AhR agonists) with nondioxin like PCBs or mixtures in prepubertal and adult female rats (Chu et al., 2001; van Birgelen et al., 1994b; van der Kolk et al., 1992), mice (De Jongh et al., 1993), and rabbits (Serabjit-Singh et al., 1983). Collectively, these data suggest that the suppressive effect is a high dose phenomenon associated with the presence of AhR agonists. The suppression of CYP2B might be attributed to its complex regulatory mechanisms. In contrast to CYP1A1, which is regulated through the AhR, the expression of CYP2B and 3A are regulated through heterodimerization of the retinoid X receptor with either the nuclear orphan CAR, or the pregnane X receptor, respectively (Cai et al., 2002; Masahiko and Honakoski, 2000; Sueyoshi and Negishi, 2001; Ueda et al., 2002; Zelko and Negishi, 2000). Since protein and mRNA levels of CYP2B, but not of CYP3A2, were significantly reduced by the treatment (Table 4), it may be speculated that this suppression was mediated through the CAR pathway.

In addition to enzyme suppression, large coefficients of variation characterized BROD and PROD activities among the high dose groups (Fig. 2). Dynamic hormonal and metabolic changes occurring during development may be responsible for some variability of the results in the prepubertal rat. In fact, there is a gradual development in the expression of CYP enzymes in the fetal (Hakkola et al., 2001), and immature rat liver (Hines and McCarver, 2002; Larsen-Su et al., 2001), associated with dynamic endocrine and metabolic changes (Li and Hansen, 1997). While reasons for these differences in BROD and PROD activities among individuals are yet to be determined, some variability might be attributed to gene polymorphisms, the lack of substrate specificity (Iba et al., 2000), the presence of competitive metabolizable substrate, or suicide substrates which bind to the cytochromes and inhibit their catalytic activities (Voorman and Aust, 1987). Note that EE, a suicide substrate for CYP2B enzymes (Kent et al., 2002), decreased BROD and PROD activities, particularly in the presence of high concentrations of AhR agonists (Fig. 4).

Our studies have shown that AhRM does not affect uterine weight at 21 days of age, or the EE induced uterotrophic effect. The absence of antiestrogenic effects of AhRM on the uterus are consistent with the results from White et al. (1995) showing an absence of antiestrogenic effects of TCDD (even at high doses) on uterine tissues in prepubertal female rats. Others have reported antiestrogenic activity of numerous AhR agonists in slightly older (23–26 day old) prepubertal female rats (Chen et al., 2001; Jansen et al., 1993; Ohtake et al., 2003;
Reasons for the controversial findings are yet to be explained, but they may be related to specific AhR agonists or dosages being tested, to the selection of the potent EE instead of estradiol-17β as estrogenic agent, to the circulating level of estradiol-17β that is known to differ even in age-matched prepubertal rats, and to developmental delays prior to the establishment of crosstalk between the AhR and ER. It has been demonstrated that the antiestrogenic activity of AhR agonists is largely mediated through crosstalk pathways between the ER and AhR (Chen et al., 2001; Safe, 1999; Zacharewski and Safe, 1998). The regulation of AhR is tissue-specific, with a developmental down-regulation of the AhR protein in the rat prostate occurring from postnatal day 7 to 21 (Sommer et al., 1999). We are not aware of similar observations made from the prepubertal female reproductive tract. However, the estrogenic environment, which is at the nadir in the 21-day-old female rat (Li and Hansen, 1997), is likely to influence the expression of the AhR. Chaffin et al. (2000) showed changes in the expression of the AhR in the liver and the ovary during the reproductive cycle of Sprague-Dawley rats, with high levels being present during the estrogenic phase (early proestrus), but suggested that estradiol-17β was not the key regulator of this process. Others have reported that estradiol-17β enhances AhR expression and CYP 1A1 induction by TCDD in the immature (Petroff et al., 2001a) and adult female rat (Sarkar et al., 2000). The sensitivity of the uterine response to detect antiestrogenic compounds should also be investigated in adult ovariecotomized rats (Kanno et al., 2001) or immature mice (Newbold et al., 2001; Ohtake et al., 2003; Padilla-Banks et al., 2001).

Despite the lack of antiestrogenic effects detected in uterine tissues from 21-day-old female rats, the 1000×-AhRM reduced ERα mRNA levels by 87% in the liver (Table 4), but had no effects in the uterine tissue (oil: n = 6, 1.12 ± 0.11; 1000×: n = 7, 1.31 ± 0.11 attomM ERα/50mg β-actin mRNA; data added during revision). Similar observations were made in adult rats seven days after exposure to TCDD, in which the density of the ER protein decreased by 92% in the liver, but only slightly in the uterus (Hruska and Olson, 1989). Perhaps the lower sensitivity of the uterus to the effects of the AhRM may be attributed to pharmacodynamic differences between the 21-day-old uterus and the liver.

The results from testing the effects of 1000×-AhRM with EE provided indications of additive and nonadditive/antagonist effects. AhR agonists are known to induce immunotoxicity (Lin et al., 2001; Tryphonas et al., 2001) and, as expected, the spleen and thymus weights were slightly reduced in AhRM treated rats (Tables 2, 3, and 5). Coadministration of AhRM with EE elicited additive effects and further decreased spleen weight, an observation supported by the immunomodulatory role of endogenous (Ito et al., 2002; Karpuzoglou-Sahin et al., 2001) and exogenous estrogens (genistein, methoxychlor; Guo et al., 2002). This additive effect contrasts with the reported antiestrogenic effects of AhR agonists, and highlights organ differences in chemical interactions. Although the AhRM reduced adrenal weight and EE counteracted this response, these treatments had no effect on serum corticosterone. However, TCDD has been reported to inhibit the adrenal cytochrome P450 side-chain cleavage (Harvey et al., 1999) and adrenal steroidogenesis (Goldman and Yawetz, 1992). In our experiment, all necropsies were performed in the morning, a time at which corticosterone levels are at their lowest. It is possible that collecting serum samples at a later time of the day, when corticosterone levels peak, might have facilitated the detection of treatment effects. The compensatory effect of EE on the AhR-induced adrenal weight loss might be explained by the fact that estradiol stimulates adenocorticotropin secretion (Harvey et al., 1999), increases adrenal blood flow and lipid accumulation, and thus adrenal weight (Hinson and Raven, 1999). The 1000×-AhRM and EE treatments reduced kidney weights (Table 5), in line with the observations that estrogens exacerbate nephrotoxicity and even induce kidney tumorigenesis (Devanesan et al., 2001; Healing, 1999; Nakamura et al., 2001). Adrenals and kidneys interact through the renin-angiotensin system (Healing, 1999), and perhaps interference with this system is linked to changes in both organ weights. Brain weight was slightly increased by EE (Table 5), perhaps as a consequence of direct beneficial actions of estrogens on the nervous system (Matsson, 1999), and/or indirectly via estrogen stimulation of the thyroid system (Croissandeau et al., 1996; Kimura et al., 1994; Lisboa et al., 1997; Schomburg and Bauer, 1997), which is a regulator of normal brain development (Howdeshell, 2002; Zoeller et al., 2002). Stimulation of the thyroid system by EE is supported by our results showing that 1000×-AhRM reduced serum thyroxin, but this effect was attenuated by EE (Fig. 4). A reduction in serum thyroxin induced by the AhRM was expected since multiple components of the thyroid system are vulnerable to the effects of AhR agonists (Brouwer et al., 1998b; Khan et al., 2002; Vansell and Klaassen, 2002a,b).

The lowest level of exposure achieved in the current experiment (AhRM-1×) represents the average exposure level of a breast fed infant during its first 24 days of life (Table 1). This was based on the average milk fat levels of AhR agonists present in Caucasian breast milk from southern Quebec during 1989–1990 (Dewailly et al., 1992). In general, these levels were similar to those reported in studies from the U.S., Europe (Dewailly et al., 1996; Schecter and Piskac, 2001; WHO, 1996), and Canada (Newsome and Ryan, 1999), but 2 to 10 times smaller than those measured from Inuit women living in Arctic Québec (Dewailly et al., 1992). The use of the current study for risk assessment should consider three factors that may contribute to overestimating possible risks in the general population. First, the levels of AhR agonists in breast milk have been declining (Craan and Haines, 1998; Norén and Meironyte, 2000; Solomon and Weiss, 2002; WHO, 1996), possibly by half every 9.6 years (WHO, 1996). Second, dosages were based on mean concentrations (Table 1) and this data is inherently not normally distributed, which implies that a large proportion of samples had much lower residue levels than
the mean. Finally, there is a wide range of beneficial effects of breastfeeding (American Academy of Pediatrics, 1997; Haller and Simper, 1999; McVea et al., 2000; Oddy, 2001; Plutta et al., 2000), and it is suspected to counteract adverse neurological effects presumed to occur from in utero exposure to organochlorines (Ribas-Fito et al., 2003).

In summary, the highlights of this article are (1) the AhRM administered by gavage during the postnatal period had no effects in the rat at a dose ten times the amount consumed by an infant over its first 24 days of life; (2) despite reports that AhR agonists are antiestrogenic, the AhRM had no effects on basal or EE-stimulated uterine growth; (3) while the AhRM increased EROD activity, it suppressed CYP2B activity, protein, and mRNA levels; (4) there were indications of agonistic and nonadditive/antagonistic interactions between the AhRM and EE; and (5) changes in AhR-dependent endpoints (EROD, body weights, serum thyroxin) were associated with TCDD-TEQ doses (1000×-AhRM > PCDDs > PCBs > PCDFs), even when testing complex mixtures.

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